Primary structure of the glycans from mouse serum and milk transferrins

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A 'serotransferrin-like' protein was purified from mouse milk. This serotransferrin cross-reacts immunologically with the serotransferrin isolated from mouse plasma and not with the mouse lactotransferrin (lactoferrin). Sugar analysis of the three transferrins, i.e. serotransferrin, milk 'serotransferrin-like' protein and lactotransferrin, revealed that the major difference between the glycan primary structure of mouse serotransferrin and those of mouse milk 'serotransferrin-like' protein and lactotransferrin concerns essentially the presence of one fucose residue in the last two proteins. For structural determination, the *N*-glycosidically linked glycans were released from the protein by a reductive cleavage of the oligosaccharide-protein linkage under strong alkaline conditions. The primary structure of the released oligosaccharide alditols was determined by methylation analysis and 400 MHz ¹H-n.m.r. spectroscopy. The oligosaccharide alditols released from milk 'serotransferrin-like' protein and lactotransferrin were identical and were identified as disialylated biantennary glycans of the *N*-acetyl-lactosamine type with a fucose residue α -1,6-linked to the *N*-acetylglucosamine residue conjugated to the peptide chain and having the following primary structure: NeuAc(α 2-6)Gal(β 1-4)GlcNAc(β 1-2)Man(α 1-3)[NeuAc(α 2-6)Gal(β 1-4)GlcNAc(β 1-2)Man(α 1-6)]Man(β 1-4)GlcNAc(β 1-4)[Fuc(α 1-6)]GlcNAc(β 1-N)Asn. The serotransferrin glycan has the same primary structure but is only partially fucosylated (10–15%).

INTRODUCTION

The family of transferrins, which are defined as iron-binding glycoproteins, is composed of four groups of components that are immunologically and structurally different: those of ovotransferrins from bird egg-white, lactotransferrins present in most mammalian milks and in leucocytes, melanotransferrins characterized in human melanoma cells and serotransferrins from serum of all vertebrates and of some invertebrates (for reviews see Williams, 1985; Montreuil *et al.*, 1985).

In addition, proteins immunologically identical with serotransferrins have been characterized in different biological fluids. As their chemical identity with the serotransferrins has not yet been established, we propose to call these proteins 'serotransferrin-like' proteins.

Variable proportions of a 'serotransferrin-like' protein and of lactotransferrin (also called lactoferrin) have been found in mammalian milk wheys (Masson *et al.*, 1965). For instance, in human and cow wheys the major iron-binding protein has been identified as lactotransferrin (Montreuil & Mullet, 1980; Montreuil *et al.*, 1960; Groves, 1960), whereas in rabbit milk whey the major iron-binding protein has been identified as a 'serotransferrin-like' protein (Baker *et al.*, 1968). In the milk whey of other animal species, such as the guinea pig and the mouse, lactotransferrin and 'serotransferrin-like' protein have been characterized (Masson & Heremans, 1971; Kinkade *et al.*, 1976).

A lactotransferrin has been highly purified by Kinkade *et al.* (1976) and by Sawatzki & Kubanek (1983) from

mouse milk, and no immunological cross-reaction was observed between mouse lactotransferrin and serotransferrin (Kinkade *et al.*, 1976).

In the present paper we describe the isolation of the 'serotransferrin-like' protein from mouse milk and we compare the glycan primary structure of this protein with those of mouse lactotransferrin and serotransferrin. This analysis was performed in order to relate the glycan structure of the transferrins and their site of biosynthesis.

EXPERIMENTAL

Materials

Mouse plasma was collected by cardiac puncture from N.M.R.I. mice, Süddeutsche Versuchstierfarm, Tuttlingen, Germany. The heparinized samples were pooled. Mouse milk was obtained from anaesthetized mice by using the milking device described by Hoffmann *et al.* (1982). The mouse milk samples were pooled and stored frozen.

Antibodies to mouse serotransferrin and to mouse milk lactotransferrin were either obtained from Cappel Laboratories (Cochranville, PA, U.S.A.) or raised in rabbits and goats by the method previously described (Sawatzki *et al.*, 1981).

Bio-Gel P-2 was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Sephadex G-25, CM-Sephacrose CL-6B, DEAE-Sephacrose CL-6B, Sephacryl S-300, Heparin-Sephacrose, the Sephadex G-25 (medium grade) PD-10 column as well as the Mono Q HR5/5 and the

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Superose 12 columns for fast protein liquid chromatography were obtained from Pharmacia (Uppsala, Sweden).

$^2\text{H}_2\text{O}$ (99.95 atom % ^2H) was from Commissariat à l'Energie Atomique (Saclay, France). All reagents were of the highest purity and all solvents were redistilled before use.

Isolation of transferrins

'Serotransferrin-like' protein and lactotransferrin were isolated from mouse milk. The whey was prepared by delipidation and casein precipitation at pH 4.6. In a typical experiment, after centrifugation at 3000 *g* for 30 min, 100 ml of whey was saturated with iron by using Fe^{3+} -nitrilotriacetate reagent (Sawatzki *et al.*, 1981). The supernatant was desalted on a Sephadex G-25 column (73 cm $\times$ 5 cm). The effluent (350 ml) was applied to a CM-Sepharose CL-6B column (9.5 cm $\times$ 5 cm), which was further eluted with 50 mM-NaCl/20 mM-Mops/NaOH buffer, pH 6.5, and then with a salt gradient (50–700 mM-NaCl) in 20 mM-Mops buffer, pH 6.5, as described previously by Sawatzki & Kubanek (1983). The non-adsorbed fraction (320 ml) containing the 'serotransferrin-like' protein was further purified on a DEAE-Sepharose CL-6B column (15.5 cm $\times$ 5 cm) equilibrated with 25 mM-Tris/HCl buffer, pH 7.8. Elution of this column was performed by a salt gradient (50–500 mM-NaCl) in the same buffer. The pool (170 ml) from the CM-Sepharose CL-6B column containing the lactotransferrin, starting at 455 ml of the gradient, was desalted on a Sephadex G-25 column (73 cm $\times$ 5 cm) and purified on a heparin-Sepharose column (14 cm $\times$ 1.6 cm) in 50 mM-NaCl/25 mM-Mops/NaOH buffer, pH 6.5. Elution was performed with an NaCl gradient (50–500 mM) in 25 mM-Mops buffer, pH 6.5.

Mouse 'serotransferrin-like' protein and lactotransferrin were concentrated by freeze-drying and further purified by gel chromatography on a Sephacryl S-300 column (95 cm $\times$ 2.5 cm) in 200 mM-Tris/HCl buffer, pH 7.8, and finally desalted on a Sephadex G-25 column in 10 mM-ammonium acetate, pH 7.8. The eluates were freeze-dried for storage.

Mouse serotransferrin was isolated by the method described by Sawatzki *et al.* (1981) or by fast protein liquid chromatography on Mono Q HR 5/5 and Superose 12 columns as follows. Mouse plasma was desalted on a Sephadex G-25 (medium grade) PD-10 column equilibrated with 50 mM-Tris/HCl buffer, pH 8.6, and frozen until use. Before injection on to the Mono Q HR 5/5 column, the mouse plasma was dialysed overnight at 4 °C against 50 mM-Tris/HCl buffer, pH 8.6 (buffer A), centrifuged and filtered on a 0.22 μm -pore-size Millipore filter. Chromatography was developed with buffer A containing increasing concentrations of NaCl. The NaCl gradients were obtained by mixing buffer A with buffer B (500 mM-NaCl in buffer A). Serotransferrin was eluted at a flow rate of 1 ml/min in a major peak at a concentration of 75 mM-NaCl.

The serotransferrin fractions collected from several identical runs were pooled and concentrated with an immiscible CX-10 Millipore unit and submitted to gel-filtration chromatography on a Superose 12 column equilibrated with 500 mM-NaCl/50 mM-Tris/HCl buffer, pH 8.6, at a flow rate of 0.2 ml/min. The fractions containing the serotransferrin were pooled, dialysed against distilled water and freeze-dried.

Immunological and electrophoretical techniques

The purity of the three mouse transferrins was checked by SDS/polyacrylamide-gel electrophoresis with the Phast-system and Phast-gels (gradient 10–15) under the conditions given in the technique files nos. 110 and 200 published by Pharmacia.

Rabbit or goat antibodies to serotransferrin and lactotransferrin were prepared by the procedure of Harboe & Ingild (1973). The presence of identical epitopes in the three transferrins was analysed by the immunodiffusion technique of Ouchterlony (1949).

Transferrins and lactotransferrin were determined by rocket immunoelectrophoresis as described by Laurell (1966) and modified by Weeke (1973).

Reductive cleavage of the glycan-protein linkage

The glycans of the three transferrins were cleaved from the protein core under strong alkaline conditions in the presence of NaBH_4 (Lee & Scoocca, 1972). Briefly, the cleavage was performed by treating 0.2 μmol of each transferrin with 0.5 ml of a solution containing 1 M- NaBH_4 /1 M-NaOH at 100 °C for 6 h. The reaction was stopped by addition of conc. acetic acid to neutralize the solutions, which were then applied to a Bio-Gel P-2 column (0.8 cm $\times$ 40 cm) and eluted with distilled water. The oligosaccharide alditols were monitored by dotting each collected fraction on a silica-gel thin-layer sheet and by staining with an orcinol/ H_2SO_4 reagent. The oligosaccharide alditols were re-*N*-acetylated by treatment with pyridine/acetic anhydride according to the procedure of Reading *et al.* (1978) and purified by gel filtration on a Bio-Gel P-2 column (0.8 cm $\times$ 40 cm). The oligosaccharide alditols were eluted with distilled water and freeze-dried.

Carbohydrate composition

Percentage composition in neutral sugars, hexosamines and sialic acids was determined by colorimetric micro-procedures described by Spik & Montreuil (1963). The monosaccharide molar proportions present in the glycan moiety of the three transferrins and in the oligosaccharide alditols were determined after methanolysis (Zanetta *et al.*, 1972) and g.l.c. of the trimethylsilylated methylglycosides on a capillary CP SIL 5 CB column (25 m $\times$ 0.25 mm) in accordance with Kamerling *et al.* (1975). The reduced-end residues were characterized as alditols. The *N*-acetylneuraminic acid residue was identified by g.l.c.-m.s.

Methylation analysis of the oligosaccharides

The oligosaccharide alditols were permethylated by the method of Hakomori (1964) with the lithium methanesulphanyl carbanion reagent (Paz Parente *et al.*, 1985). The permethylated oligosaccharides were subjected to methanolysis, and the partially methylated methyl glycosides were peracetylated with pyridine/acetic anhydride (1:1, v/v) at 37 °C for 24 h. The peracetylated and permethylated methyl glycosides were then separated by g.l.c. in a capillary column (25 m $\times$ 0.3 mm) coated with OV-101 under the conditions described by Fournet *et al.* (1981). The monosaccharide derivatives were also identified by g.l.c.-m.s. in a RIBERMAG R 10.10 mass spectrometer (Riber, Rueil-Malmaison, France) coupled to the data system Sydar 121.

Table 1. Summary of the purification procedure for mouse milk 'serotransferrin-like' protein

For details see the text.

Fraction	Volume (ml)	Protein		Transferrin		Purity (%)	Recovery (%)
		(mg/ml)	(g)	(mg/ml)	(g)		
Whey	100	61	6.10	5.1	0.51	8.4	100
Desalted whey	245	19	4.65	2.0	0.49	10.5	96
CM-Sepharose fraction	450	7	3.15	1.1	0.49	15.7	96
DEAE-Sepharose fraction	130	3.5	0.46	2.8	0.36	80	71
Sephacryl S-300 fraction	56	5.4	0.30	5.3	0.29	98	58

¹H-n.m.r. spectroscopy

The reduced oligosaccharides were repeatedly dissolved in ²H₂O at room temperature and at pD 7 with intermediate freeze-drying (Vliegthart *et al.*, 1983). The deuterium-exchanged oligosaccharide alditols were submitted to ¹H-n.m.r. spectroscopy performed at 400 MHz on a Bruker AM 400-WB spectrometer operating in the pulsed Fourier-transform mode and equipped with a Bruker Aspect 3000 computer at a probe temperature of 300 K (Centre Commun de Mesures, Université de Lille Flandres-Artois). Resolution enhancement of the spectra was achieved by Lorentzian-Gaussian transformation by the procedure of Ernst (1966). Chemical shifts (δ) are expressed in p.p.m. downfield from the signal for internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured by reference to internal acetone ($\delta = 2.225$ p.p.m.) with an accuracy of 0.002 p.p.m.

RESULTS**Isolation of transferrins**

Milk 'serotransferrin-like' protein was easily separated from lactotransferrin on the CM-Sepharose CL-6B column. In fact, the 'serotransferrin-like' protein, which possesses an acidic isoelectric point, like serotransferrin isolated from serum of various species, does not bind to the cation-exchange column and was eluted from the column with other non-retained proteins by the starting buffer, i.e. 50 mM-NaCl/20 mM-Mops buffer, pH 6.5. The lactotransferrin, a basic protein, like lactotransferrins isolated from milk of different species, was retained on the CM-Sepharose column and was eluted from the column with an NaCl gradient. Purification of the 'serotransferrin-like' protein was achieved by DEAE-Sepharose CL-6B ion-exchange chromatography and gel chromatography on Sephacryl S-300. The complete isolation procedure is summarized in Table 1, giving the purity and recovery for each step. Recovery of 'serotransferrin-like' protein was about 60%, as calculated from the content in the milk, the amounts of lactotransferrin and 'serotransferrin-like' protein present in mouse milk were determined to be 1 mg/ml and 3 mg/ml respectively. Both transferrins were characterized by their ability to bind two iron ions.

Fast protein liquid chromatography provides a fast, highly reproducible, fractionation of serotransferrin with a recovery of more than 70%. Moreover, this procedure can be scaled up.

Fig. 1. SDS/polyacrylamide-gradient-gel electrophoresis of transferrins: Coomassie Blue staining patterns

Lane 1, mouse milk 'serotransferrin-like' protein; lane 2, mouse serotransferrin; lane 3, mouse milk lactotransferrin; lane 4, human milk lactotransferrin; lane 5, marker proteins phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soya-bean trypsin inhibitor (M_r 21000) and α -lactalbumin (M_r 14400).

The isolated mouse transferrins were homogeneous, as assessed by SDS/polyacrylamide-gel electrophoresis (Fig. 1). The M_r values of the three mouse transferrins deduced from their electrophoretic migration were about 78000 (Sawatzki *et al.*, 1981). The human lactotransferrin has a higher M_r than those of all mouse transferrins.

Analysis of the polypeptide chain of transferrins

Serotransferrin and milk 'serotransferrin-like' protein totally cross-reacted with antibodies raised against the serotransferrin. In contrast, lactotransferrin and serotransferrin or 'serotransferrin-like' protein did not cross-react with anti-lactotransferrin or anti-serotransferrin antibodies (Fig. 2). This result suggests that the antigenic

Fig. 2. Radial immunodiffusion pattern of mouse milk lactotransferrin (m-Lf), mouse serotransferrin (s-Tf) and mouse milk 'serotransferrin-like' protein (m-Tf) against the antibodies to mouse serotransferrin (anti-Tf) from rabbit and to mouse milk lactotransferrin (anti-Lf) from goat

determinants of the polypeptide chain from serotransferrin and milk 'serotransferrin-like' protein are identical or very similar and that they differ from those of lactotransferrin. Preliminary results concerning the amino acid sequences of serotransferrin and milk 'serotransferrin-like' protein have shown that the *N*-terminal parts of both proteins are identical and differ from the *N*-terminal amino acid sequence of mouse lactotransferrin (Y. Leclercq, G. Sawatzki, P. Maes & G. Spik, unpublished work).

Determination of the primary structure of oligosaccharide alditols

Carbohydrate composition. When the native transferrins were examined for their percentage carbohydrate compositions, similar values (3.2–3.5 %) were obtained. The pure oligosaccharide alditols released by the alkaline-reductive treatment from the three transferrins were obtained in a good yield (75 %) by gel filtration on a Bio-Gel P-2 column, and fully re-*N*-acetylated. Their molar carbohydrate content, given in Table 2, shows that the proportions of sialic acid, galactose, mannose and fucose are similar to those found in the three transferrins, indicating that no degradation of the monosaccharides occurred during the alkaline treatment. Under g.l.c.

analysis conditions, the trimethylsilylated derivative of *N*-acetylglucosaminitol was separated from the trimethylsilylated derivative of methyl *N*-acetylglucosaminide. The results reported in Table 2 also show that the major difference between the carbohydrate molar proportions of the three transferrins concerns the number of fucose residues. In fact, serotransferrin glycan was characterized by the lack of one fucose residue compared with the milk 'serotransferrin-like' glycan.

Methylation analysis. The relative proportions of the methylated derivatives of sialic acid, neutral sugars and *N*-acetylhexosamine taken from the mass fragmentography scans and combined by using the data of the sugar compositions are given in Table 3. The presence (i) of one residue of 2,4-di-*O*-methyl mannoside for two residues of 3,4,6-tri-*O*-methyl mannoside, (ii) of two residues of 2,3,4-tri-*O*-methyl galactoside for two residues of 4,7,8,9-tetra-*O*-methyl derivative of *N*-acetyl-*N*-methylneuraminic acid and (iii) of three residues of the 3,6-di-*O*-methyl derivative of *N*-acetylglucosamine in the oligosaccharide alditols is interpreted in terms of the presence of glycans possessing a biantennary structure of the disialylated *N*-acetyl-lactosamine type in the three transferrins.

Occurrence of methyl derivatives of *N*-acetylglucosaminitol in the three oligosaccharide alditols indicates that the *N*-acetylglucosamine residue is located at the reducing-end position of the cleaved oligosaccharides and that this amino sugar is involved in the *N*-linkage to the protein core of the three transferrins. The characterization of 0.5 residue of 1,3,5,6-tetra-*O*-methyl-*N*-methylacetylglucosaminitol derivative in the oligosaccharide alditol from serotransferrin and of 0.4 residue of 1,3,5-tri-*O*-methyl-*N*-methylacetylglucosaminitol derivative in the oligosaccharide alditols from 'serotransferrin-like' protein and lactotransferrin shows that the *N*-acetylglucosamine linked to the protein is substituted at the C-6 position in 'serotransferrin-like' protein and in lactotransferrin.

400 MHz ¹H-n.m.r. spectroscopic analysis. The 400 MHz ¹H-n.m.r. spectra of the three oligosaccharide alditols were recorded for neutral ²H₂O solutions at a probe temperature of 27 °C. Relevant n.m.r. parameters are listed in Table 4. Analysis of the spectrum of the oligosaccharide alditol released from serotransferrin shows that a structure of the biantennary type can be

Table 2. Molar carbohydrate composition of the mouse serotransferrin, 'serotransferrin-like' protein and lactotransferrin and of the oligosaccharide alditols released by reductive alkaline cleavage of the glycan-protein linkage

The molar proportions were calculated on the basis of 3 mannose residues.

Compound	Molar carbohydrate composition					
	NeuAc	Fuc	Gal	Man	GlcNAc	GlcNAc-ol
Serotransferrin	1.8	0.1	2.5	3.0	4.0	0
Serotransferrin oligosaccharide alditol	1.5	0.1	2.2	3.0	2.8	Traces
'Serotransferrin-like' protein	1.7	1.3	2.5	3.0	4.1	0
'Serotransferrin-like' protein oligosaccharide alditol	1.7	1.2	2.1	3.0	2.8	Traces
Lactotransferrin	1.8	1.3	2.4	3.0	3.6	0
Lactotransferrin oligosaccharide alditol	1.7	1.1	2.4	3.0	2.5	Traces

Table 3. Molar proportions of monosaccharide methyl ethers present in the methanolysates of permethylated oligosaccharide alditols released by reductive alkaline cleavage from the protein of mouse serotransferrin, 'serotransferrin-like' protein and lactotransferrin

The molar proportions were calculated on the basis of 2 residues of (3,4,6-)Me₃Man per molecule of oligosaccharide alditol.

Monosaccharide methyl ether	Molar proportions in reduced oligosaccharides isolated from		
	Serotransferrin	Milk 'serotransferrin-like' protein	Milk lactotransferrin
(3,4,6-)Me ₃ Man	2	2	2
(4,7,8,9-)Me ₄ NeuAcMe	2.4	2.1	2.1
(2,3,4-)Me ₃ Fuc	0.1	0.9	0.8
(2,3,4-)Me ₃ Gal	1.7	2.1	2.0
(2,4-)Me ₂ Man	1.1	1.1	1.0
(3,6-)Me ₂ GlcNAcMe	3.4	2.8	2.8
(1,3,5,6-)Me ₄ GlcNAcMe-ol	0.5	0	0
(1,3,5-)Me ₃ GlcNAcMe-ol	0	0.4	0.4

Table 4. ¹H chemical shifts of structural reporter groups of constituent monosaccharides for the oligosaccharide alditols released from the three mouse transferrins

Compounds are represented by schematic structures (cf. Vliegenthart *et al.*, 1983): ●—, GlcNAc; ◆—, Man; ■—, Gal; □—, Fuc; ○—, NeuAc. The numbering of sugar residues follows that shown in Fig. 4. Abbreviation: N.D., not determined.

		Chemical shift (p.p.m.)		
Reporter group	Residue	Serotransferrin	Milk 'serotransferrin-like' protein	Lactotransferrin

1	GlcNAc	4.642	N.D.	N.D.
	Man-3	4.782	4.785	4.777
	Man-4	5.134	5.134	5.134
	Man-4'	4.946	4.939	4.938
	GlcNAc-5	4.605	4.603	4.603
	GlcNAc-5'	4.605	4.603	4.603
	Gal-6	4.443	4.443	4.442
	Gal-6'	4.443	4.443	4.442
	Fuc	—	4.895	4.895
2	Man-3	4.256	4.257	4.255
	Man-3	4.256	4.257	4.255
	Man-4	4.198	4.198	4.197
	Man-4'	4.119	4.111	4.111
3 _{ax.}	NeuAc(2-6)	1.717	1.718	1.717
3 _{eq.}	NeuAc(2-6)	2.671	2.678	2.676
5	Fuc	—	4.071	4.072
CH ₃	Fuc	—	1.225*	1.226*
Ac	GlcNAc-1-ol	2.056	2.057	2.056
	GlcNAc-2	2.083	2.090	2.093
			2.094	2.090
	GlcNAc-5	2.071	2.071	2.071
	GlcNAc-5'	2.065	2.066	2.066
	NeuAc	2.030	2.030	2.030

* A doubling of this doublet (δ = 1.225 and 1.221 p.p.m.) was observed upon resolution-enhancement of the spectrum. As yet this feature could not be explained.

Fig. 3. Resolution-enhanced 400 MHz ^1H -n.m.r. spectra ($^2\text{H}_2\text{O}$, 27 °C) of oligosaccharide-alditols isolated from mouse lactotransferrin (a), 'serotransferrin-like' protein (b) and serotransferrin (c)

The relative-intensity scale of the *N*-acetyl proton region differs from that of the other parts of the spectrum, as indicated. From the signals marked by asterisks (*), especially from the intensity of CH_3 of fucose at 1.225, of *N*-acetyl proton singlet of GlcNAc-2 at 2.090, of H-5 of fucose at 4.071 and of anomeric proton of fucose at 4.895, it can be inferred that sample (c) contained a small proportion of fucose.

deduced from the chemical-shift values of the H-2 atoms of the three mannose residues (for Man-3 $\delta = 4.256$ p.p.m., for Man-4 $\delta = 4.198$ p.p.m. and for Man-4' $\delta = 4.119$ p.p.m.) (Vliegthart *et al.*, 1983; Cahour *et al.*, 1984) (Fig. 3).

The spectrum indicates the occurrence of two *N*-acetylneuraminic acid residues, as shown by the intensity of their *N*-acetyl signals. From the set of chemical-shift values of their structural reporter groups (H-3_{ax}, $\delta = 1.717$ p.p.m., H-3_{eq}, $\delta = 2.671$ p.p.m. and *N*-acetyl protons $\delta = 2.030$ p.p.m.), the linkage of *N*-acetylneuraminic acid is characterized as (α 2-6)Gal. The chemical-shift values of H-1 of Man-4 $\delta = 5.134$ p.p.m. and Man-4' $\delta = 4.946$ p.p.m. demonstrate the sialylation of the two antennae on Gal-6 and Gal-6'. The *N*-acetyl signals of GlcNAc-5 and GlcNAc-5', resonating at $\delta = 2.071$ p.p.m. and at $\delta = 2.065$ p.p.m. respectively, are typical of sialylated antennae. The chemical-shift values of the H-1 atom of GlcNAc-2 and the *N*-acetyl signals of GlcNAc-2 and *N*-acetylglucosaminitol are at $\delta = 4.642$ p.p.m., $\delta = 2.083$ p.p.m. and $\delta = 2.056$ p.p.m. respectively. The low-intensity structural reporter group signals for fucose (H-1 $\delta = 4.895$ p.p.m. and CH-3 $\delta = 1.225$ p.p.m.) are typical for fucose α -1,6-linked to *N*-

acetylglucosaminitol and show that some heterogeneity exists (about 10–15%).

In summary, the main component of this oligosaccharide alditol fraction is a classic biantennary structure having *N*-acetylneuraminic acid α -2,6-linked to Gal-6 and Gal-6' without fucose α -1,6-linked to *N*-acetylglucosaminitol.

Analysis of the spectra of the oligosaccharide alditols released from 'serotransferrin-like' protein and lactotransferrin demonstrates the presence of disialyl bi-antennary oligosaccharide alditol of the *N*-acetyl-lactosamine type (Table 4). The spectra show a very close resemblance with those already reported (Fig. 3). The only difference is the presence of one residue of fucose α -1,6-linked to *N*-acetylglucosaminitol; this was confirmed by the set of chemical-shift values for the fucose reporter group (H-1 $\delta = 4.895$ p.p.m., H-5 $\delta = 4.071$ p.p.m. and CH-3 $\delta = 1.225$ p.p.m.). It may be noticed that these values are considerably altered when compared with those of glycopeptides. This is due to the presence of GlcNAc-ol instead of GlcNAc(β 1-N)Asn.

The *N*-acetyl singlet at $\delta = 2.056$ or 2.057 p.p.m. is ascribed to the GlcNAc-1-ol residue. The *N*-acetyl methyl protons of GlcNAc-2, β -1,4-linked to GlcNAc-

Fig. 4. Primary structure of the oligosaccharide alditols released by reductive alkaline cleavage of the glycan-protein linkage of mouse serotransferrin (a) and of mouse milk 'serotransferrin-like' protein and lactotransferrin (b)

ol, were found to resonate at $\delta = 2.090$ p.p.m. and 2.094 p.p.m. (the value of H-1 atom of GlcNAc-2 could not be determined because the doublet is hidden under the $^1\text{HO}^3\text{H}$ line). The deviations of these values are attributed to the presence of fucose α -1,6-linked to GlcNAc-1-ol.

In summary, the oligosaccharide alditols isolated from 'serotransferrin-like' protein and lactotransferrin are identical and possess a fucosylated biantennary structure having *N*-acetylneuraminic acid α -2,6-linked to Gal-6 and Gal-6' and fucose α -1,6-linked to *N*-acetylglucosaminitol.

DISCUSSION

Kinkade *et al.* (1976) first observed that mouse milk whey contains two immunologically distinct iron-binding proteins. In the present work the 'serotransferrin-like' protein was found to be present in the milk at a concentration 3-fold higher than the lactotransferrin. The biological significance of this high concentration, as well as the presence of 'serotransferrin-like' protein only in rabbit milk (Baker *et al.*, 1968), is, at the moment, not well understood. These observations are curious, especially as mouse 'serotransferrin-like' protein, in contrast with mouse milk lactotransferrin, seems to play no role in the iron transfer on the mouse brush-border-membrane vesicles (W. Hu, J. Mazurier, G. Sawatzki, J. Montreuil & G. Spik, unpublished work).

The mouse 'serotransferrin-like' protein was purified to homogeneity as judged by the polyacrylamide-gel electrophoresis under denaturing conditions. Moreover, from its electrophoretic mobility, the M_r of the single polypeptide chain has been estimated to be $78\,000 \pm 2000$, a value similar to those of mouse serotransferrin and lactotransferrin (Fig. 1). On the basis of the M_r value and of the sugar content, we can assume that the three transferrins possess a single glycan of M_r 2500.

Under the strong alkaline-reductive conditions of cleavage of the GlcNAc-Asn linkage in accordance with Lee & Scoocca (1972), the oligosaccharides were completely released from the protein of the three transferrins. As indicated in Table 2, the recovery of trimethylsilylated

derivative of *N*-acetylglucosaminitol was very low. The decrease in the amount of *N*-acetylglucosaminitol was not due to complete degradation of the *N*-acetylglucosamine linked to asparagine, since by methylation 0.5 and 0.4 residue of methylated derivatives of *N*-acetylglucosaminitol were identified.

On the basis of methylation data and of ^1H -n.m.r. analysis the complete primary structure of the three reduced oligosaccharides has been established as a disialylated biantennary *N*-acetyl-lactosaminic structure (Fig. 4). However, this structure contains in the oligosaccharide alditol released from lactotransferrin and 'serotransferrin-like' protein one fucose residue α -1,6-linked to the *N*-acetylglucosamine reduced to *N*-acetylglucosaminitol. Characterization by ^1H -n.m.r. analysis, in the oligosaccharide alditol fraction released from mouse serotransferrin, of 10–15 % oligosaccharide alditol containing an α -1,6-linked fucose residue indicates that fucosylation of serotransferrin glycan may occur in liver. This fucosylation is, however, less important than that in the mammary gland.

These results show that the carbohydrate structure of mouse serotransferrin is identical with that of human (Spik *et al.*, 1975) and of rabbit (Leger *et al.*, 1978) serotransferrins, and mouse lactotransferrin possesses the same glycan structure as that of human lactotransferrin (Spik *et al.*, 1982). However, it has to be noted that human lactotransferrin also contains glycans with two fucose residues, one α -1,6-linked and the other α -1,3-linked to an *N*-acetylglucosamine residue. In addition, human serotransferrin and lactotransferrin contain two glycans linked to the polypeptide chain compared with one glycan for mouse serotransferrin and lactotransferrin. This can be observed as an apparent higher M_r of the human lactotransferrin in the SDS/polyacrylamide-gel electrophoresis (Fig. 1).

The site of biosynthesis of mouse milk 'serotransferrin-like' protein is not yet known. It could be either the liver or the mammary gland. Taking into account the specificity of the glycosyltransferases and particularly that of the fucosyltransferase, we suggest that most probably the biosynthesis of the milk 'serotransferrin-like' glycan occurs in the mammary gland. Indeed, the

fucosyltransferase, also named GDP-L-fucose:2-acetamido-2-deoxy- β -glucoside 6- α -L-fucosyltransferase (Longmore & Schachter, 1982), transfers fucose residues to substrates with biantennary glycans containing the GlcNAc(β 1-2)Man(α 1-3) sequence at the non-reducing position. Elongation of antennae by addition of Gal(β 1-4) or NeuAc(α 2-6)Gal(β 1-4) sequences prevents enzyme action (Schachter *et al.*, 1982). According to these observations, the serotransferrin glycan would have either to be desialylated and degalactosylated during the transfer from the plasma to the mammary gland before being fucosylated, and then re-galactosylated and re-sialylated or, more probably, to be completely biosynthesized, including the polypeptide chain, by the mammary gland.

This suggestion disagrees with the findings reported by Laurell & Morgan (1965) and Ezekiel (1965), which imply that the mammary gland of the rat partially works as a molecular sieve for plasma proteins, particularly as lactation progresses. Further studies are necessary to identify the site of biosynthesis of serum proteins found in mammalian milk. The biological role of the iron-binding proteins 'serotransferrin-like' protein and lactotransferrin, both present in milk, has to be elucidated, since so far only lactotransferrin appears to be involved in iron nutrition.

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